Biochimica et Biophysica Acta 1847 (2015) 1354–1361

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

Mitochondrial DNA: Radically free of free-radical driven mutations☆

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ARTICLE INFO

Article history:
Received 11 March 2015
Received in revised form 8 May 2015
Accepted 2 June 2015
Available online 4 June 2015

Keywords:
Mitochondrial DNA
mtDNA
Somatic mutations
Ageing

ABSTRACT

Mitochondrial DNA has long been posited as a likely target of oxidative damage induced mutation during the ageing process. Research over the past decades has uncovered the accumulation of mitochondrial DNA mutations in association with a mosaic pattern of cells displaying mitochondrial dysfunction in ageing individuals. Unfortunately, the underlying mechanisms are far less straightforward than originally anticipated. Recent research on mitochondria reveals that these genomes are far less helpless than originally envisioned. Additionally, new technologies have allowed us to analyze the mutational signatures of many more somatic mitochondrial DNA mutations, revealing surprising patterns that are inconsistent with a DNA-oxidative damage based hypothesis. In this review, we will discuss these recent observations and new insights into the eccentricities of mitochondrial genetics, and their impact on our understanding of mitochondrial mutations and their role in the ageing process. This article is part of a Special Issue entitled: Mitochondrial Dysfunction in Aging.

1. Introduction

All eukaryotic life on earth is derived from a highly unlikely merger between at least two independent lifeforms. Genomic evidence from eukaryotes clearly shows the signature of a merged set of genes of both archaebacterial (the ancestor of our nuclear genome) and α-proteobacterial (the ancestor of our mitochondria) origin. Curiously, mitochondria have retained their own highly reduced genomes which encode only a tiny subset of the genome of the original α-proteobacterial gene complement. This necessitates the complicated import of a large number of proteins just to translate these few peptides [1]. This has led to a large number of hypotheses, seeking to explain why a mitochondrial genome persists, and has not simply ceded complete control over to the nucleus [2–4].

Despite the estimated 2 billion years of co-evolution, the mitochondrial and nuclear genomes appear to have followed drastically different paths. Unlike the nuclear genome, the animal mitochondrial DNA (mtDNA) has typically retained the circular and densely coded character of the prokaryotic ancestor. Instead of a carefully orchestrated system by which all chromosomes are equally copied within cellular mitosis or meiosis, mitochondrial DNA is copied and turned over at random, to a tempo independent of the cell cycle. Instead of a system of paired diploidy (or tetraploidy and so on), the mitochondrial DNA is more akin to a collection of hundreds to thousands of independent but related bacterial genomes within the cell. These variations from the nucleus allow for interesting properties and issues that are unique to the organellar genomes.

In order to avoid the overwhelming complexity of eukaryotic mitochondrial genome evolution [5], it is necessary here to focus only on the Metazoa, with emphasis on mammalian mtDNA. Metazoan mtDNA encodes 11–15 protein coding genes [6,7], the large and small ribosomal RNAs, the tRNAs necessary to carry out the translation within the mitochondria, and a non-coding control region that harbours sequences important for transcription and replication of the mtDNA. Barring the few rare exceptions, the protein genes maintained on mitochondrial DNA are key components of the Oxidative Phosphorylation (OXPHOS) systems, specifically mitochondrial respiratory complexes I, III and IV and the ATP synthase (complex V). Thus, mutations of the mtDNA typically lead to a dysfunction of the respiratory chain.

It is firmly established that during ageing, a patchy distribution of cells within individuals begins to display the hallmarks of mitochondrial respiratory chain dysfunction. This pattern has been observed in many human tissues, and has been associated with the accumulation of circular mtDNAs bearing deletions or mtDNAs with accumulated point mutations. The painstaking accumulation of this data over about the past 25–30 years has been the subject of other recent reviews [8–11]. Mouse models with increased rates of mtDNA mutation [12–14] or mtDNA deletion mutations [14–16] have also shown phenotypes strikingly similar to ageing. However, there are no reports to date of models that can decrease the mutation load to test whether lower levels of mtDNA mutations can slow the onset of ageing phenotypes.

The anticipated source of mtDNA mutations was greatly influenced by Harman's free-radical theory of ageing [17,18]. Perhaps inspired by the pervious comparisons between ionizing radiation exposure and a hypothesized similarity to "oxygen poisoning" [19], Harman first proposed that oxygen radical could be responsible for cellular damage in the ageing process [17]. By 1972, he had proposed that mitochondria

☆ This article is part of a Special Issue entitled: Mitochondrial Dysfunction in Aging.

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http://dx.doi.org/10.1016/j.bbabio.2015.06.001
0005-2728/

were the primary source of cellular free radicals, and were therefore agents responsible for the free-radical based ageing [18]. This view was extended into a "vicious cycle" theory, whereby mutations accumulating over time in the mitochondrial DNA would lead to dysfunctional OXPHOS components that will go on to leak more Radical Oxygen Species (ROS) and induce more mtDNA mutation in a run-away cycle that leads to cellular impairment [20].

However, recent work that has advanced understanding of mitochondrial biology has called into question a number of the assumptions that underlie this theory. In this review, we will highlight recent research that shows mtDNA is not helpless in the face of constant free radical assault. We will also highlight recent work that characterizes these low-level somatic mtDNA mutations, and discuss what these mutational signatures tell us about mtDNA mutations during ageing. And finally, we will discuss the forces that guide these mtDNA mutations from rare and insignificant cellular occurrences to the dominant forms of mtDNA within a cell where they may induce the mitochondrial dysfunction characteristic in ageing animals.

2. mtDNA defences

2.1. DNA physical and spatial defences

One common assertion is that mitochondrial DNA is free of histones, so "naked" and vulnerable to oxidative DNA damage due to superoxide radical produced by the nearby OXPHOS system. We now know that mitochondrial DNA exists in a compacted DNA–protein complex known as the mitochondrial nucleoid [21–25]. The dual-role protein TFAM appears to be a major component of the nucleoid, but research into the exact composition and the dynamics of the protein components of nucleoids continues [26–28]. Protein-coating of the DNA is expected to act as a shield against DNA damage and enhance the stability of the double helix.

Additionally, the mitochondria are more compartmentalized than commonly appreciated. The OXPHOS complexes, which are the main sources of the free radicals, have been demonstrated to be tightly sequestered into cristae with diameters ~20 nm in size [29]. These cristae are far too small to fit the ~100 nm nucleoids [24,25], appearing to physically isolate the mtDNA from this site of free radical production. Thus, mtDNA does not sit, naked and helpless, besides the source of mitochondrial free radicals.

2.2. mtDNA repair mechanisms

A second assertion involves the lack of mtDNA repair pathways within mitochondria. This assertion likely arises from early experiments to test for nucleotide excision repair (NER) in mitochondria. Mitochondrial extracts were unable to remove most common nucleotide excision repair substrates, such as thymidine dimers [30], cisplatin intrastrand crosslinks, complex alkylation damage, and other forms of damage [31,32], inferring the absence of NER in the mitochondria. However, recent work has revealed that mitochondria can repair many other types of DNA damage. The most well-studied repair pathway found in mitochondria is base excision repair (BER), where the damaged base is recognized and removed by a specific glycosylase followed by gap tailoring, filling and ligation of the DNA. Many of the nucleus-targeted BER pathway proteins have been found to also localize into mitochondria in identical or alternatively processed forms in human cells. Two subcategories of the pathway are recognized; namely long- and short-patch BER. In short-patch BER only a few nucleotides are removed upon damage repair whereas in long-patch BER mostly 5′ blocking groups are removed, which requires removing longer stretches of DNA. The enzymes required to carry out short-patch BER have been found in mitochondria.

The first step in base excision repair is the removal of the damaged base by a specific DNA glycosylase. Six glycosylases are found in mitochondria, which are specialized in removing different damaged bases. Due to their distinct catalytic mechanisms, they require different downstream gap tailoring enzymes. Uracil DNA glycosylase I, which removes the uracil created by cytosine deamination [33,34], and the homolog of *Escherichia coli* MutY (MUTYH), which removes adenosine erroneously incorporated against 8-oxo-deoxyguanosine (8-oxo-dG) [35,36], leave behind an abasic site. In contrast, the other four glycosylases also cut the phosphodiester bond. The other glycosylases are 8-oxoguanine DNA glycosylase 1 (OGG1) and three homologs of *E. coli* glycosylases, endonuclease III (NTH1), and two endonuclease VIII-like proteins (NEIL1 and NEIL2). OGG1 removes 8-oxo-dG and possibly the ring-open form of guanine (FAPy guanosine) from double stranded DNA [37,38]. NTH1 has mostly been associated with thymine glycol removal [39–41], whereas NEIL1 is suggested to repair thymine glycol, FAPy and 5-hydroxyuracil and have some activity for 8-oxo-dG removal [42,43] and NEIL2 is involved in removing 5-hydroxyuracil and other oxidized derivatives of cytosine [44,45]. Interestingly, in in vitro assays NEIL1 and 2 are found to be most active at so-called DNA-bubble structures [46], possibly explaining the redundant damage targets of these oxidative-damage repairing glycosylases [39]. All of these glycosylases leave behind a non-ligatable ends that need to be further processed by gap tailoring enzymes apurinic/apyrimidinic endonuclease (APE1) [47] or PNKP [44,48], who have both been found in mitochondria.

In addition to short-patch repair, long-patch repair activity has been found in purified mitochondria from multiple sources such as mouse liver, kidney and human lymphoblasts [49–51]. Long-patch repair is suggested to be highly important for mitochondrial functionality, since it repairs one of the common lesions caused by oxidative damage to the sugar-phosphate backbone; 5′ deoxyribolactone [52]. In contrast to BER, DNA Mismatch repair (MMR) focuses on recognizing and repairing base–base mismatches and small loops. Mitochondrial specific MMR activity has been reported from rat mitochondrial lysates and mitoplasts of human cell lines [53,54] but the main proteins involved in nuclear MMR have not been found in mitochondria. One candidate protein with a binding activity towards mismatched DNA, YB-1, localizes to mitochondria and its knockdown decreases the MMR activity in mitochondria [55].

Animal knockout models of these repair enzymes show a range of phenotypes, from carcinogenesis (in a MUTYH knockout [56]), to knockouts that develop metabolic syndrome and obesity (OGG1 [57,58] and NEIL1 [59]). While the metabolic phenotypes are suggestive of problems with mtDNA, further work is required as the gene products are thought to function in both the mitochondria and nucleus, making the elucidation of mitochondria-specific phenotypes very difficult. Analysis of the NEIL1 knockout mice revealed increased levels of DNA damage, and the accumulation of mtDNA molecules bearing deletions, but no analyses were undertaken to determine if the mice harboured bona fide mtDNA mutations [59]. And surprisingly, some studies of knockout of OGG1 [60] or OGG1/MUTYH double knockouts [61] fail to observe enhanced accumulation of mtDNA mutations. The construction of knock-in models that disrupt only the mitochondrial or nuclear activity will be required to resolve these issues.

3. The origins of mtDNA mutations

3.1. The long road from free radical to DNA damage

Much attention has been focused on the putative role of reactive oxygen species in mitochondrial mutagenesis. ROS are thought to be more prevalent in mitochondria than the nucleus because most of the cellular ROS are produced by the mitochondrial respiratory chain, in the form of superoxide. However the path from superoxide to DNA mutation is much more indirect than implied and a complex chain of events is required to convert superoxide to a potent DNA damage molecule (Fig. 1). Superoxide is actually quite unreactive with DNA [62], and

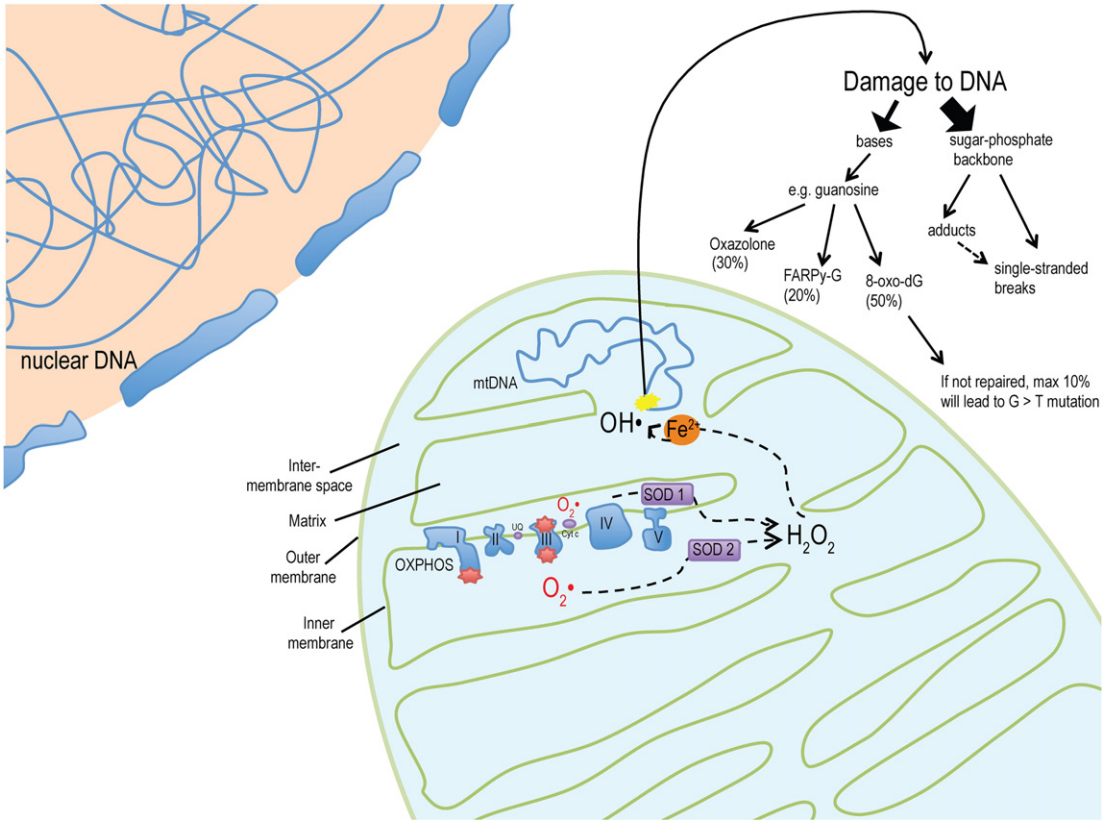

Fig. 1. The long road from free radical to mtDNA mutation; a schematic representation of oxidative damage to mitochondrial DNA and its consequences. The main sites of superoxide ($O_2^{\bullet-}$) production in OXPHOS are complexes I and III. In contrast to complex I, complex III produces superoxides to both matrix and intermembrane space. Superoxide can either transform into H_2O_2 spontaneously or with the help of SOD1 and SOD2. Neither superoxide nor H_2O_2 can efficiently attack DNA but through Fenton chemistry H_2O_2 can be converted into the highly reactive hydroxyl radical, OH*. OH* can react with either bases or the sugar-phosphate backbone leading to single-stranded breaks, abasic sites, DNA–DNA intrastrand adducts, DNA–protein crosslinks and base damage [67]. For example, the main products of guanosine base damage are oxazolone, FAPy-G and 8-oxo-dG. Whereas the highest measured mutagenic potential for 8-oxo-dG is 10% [136].

cannot cross the mitochondrial membranes [63,64]. In order to travel to and damage the mtDNA, the superoxide must be converted to H_2O_2, either spontaneously or by superoxide dismutase. H_2O_2 is able to leave mitochondria and thus cause damage to the whole cell. However, it is still an inefficient agent of DNA damage [62], and there are multiple H_2O_2 scavengers located to mitochondria to deal with H_2O_2 [65]. H_2O_2 can, however, react through Fenton chemistry with transition metals such as iron and convert to a hydroxyl radical, the most reactive ROS, whose reactivity is only restrained by diffusion [66]. A hydroxyl radical is able to react with DNA causing single stranded breaks, abasic sites, DNA–DNA intrastrand adducts, DNA–protein crosslinks and base damage [67]. Despite the H_2O_2 scavenging system there is evidence that mtDNA may be more vulnerable than nuclear DNA to H_2O_2, showing higher frequency of H_2O_2 driven lesions in cell culture systems [68]. In recent studies, it was found that H_2O_2 treatment resulted in strand breaks or abasic sites that are converted to strand breaks [69]. Possibly suggesting that instead of base modifications, single stand breaks would be the main product of oxidative damage. Thus, the relationship between free radicals and mtDNA mutations is not as straightforward as often portrayed.

The initial measurements for oxidative damage levels in mtDNA were found variably different between groups on the same tissue, mostly due to technical issues in the measurements [70]. Recently the measurements were repeated and the levels of oxidative lesions in mtDNA were found to be comparable to nuclear DNA in rat liver [71]. Some oxidative lesions such as 8-oxo-dG, FAPy guanosine and 5-hydroxyl cytosine were found at a higher frequency in nuclear DNA than mtDNA in rat liver [72]. The age of the tested animals did not have an effect on the amounts of DNA lesions in rat liver [71], suggesting that the damage does not accumulate, but was turned over or repaired.

If DNA damage is induced, we have still not moved on to a mutation. Many forms of DNA damage will induce a replication blockage instead of propagating a novel mutation [73]. One can imagine that rare replication blockages within the multi-copy mtDNA system would be a preferable outcome to mutations, which can then be propagated through the system. In vitro studies with human pol γ have revealed that replication is simply blocked upon encountering an 8-oxo-dG adduct in most instances [74]. When it did copy through, human and *Xenopus* pol γ usually inserted the appropriate C across from the adduct [74,75]. While mutations can also be introduced by incorporating damaged nucleotides, such as 8-oxo-dGTP across from and A during replication [73], pol γ has been found to strongly discriminate against the damaged base during in vitro replication assays [74]. Thus it appears that the mitochondrial polymerase itself also provides efficient protection against damage-induced mutagenesis.

Consistent with this multi-step defence against oxidative damage, recent work is beginning to demonstrate that in vivo levels of oxidative damage to mtDNA may not be of great consequence to animals. Studies in mice found no increase in mutation frequency of mtDNA mutation when both OGG1 and MUTYH are knocked out in mice [61], while the nucleus shows the hallmark increase in G to T mutagenesis in OGG1 knockout mice [76]. More surprisingly, fly models with disrupted OGG1 or SOD2 + OGG1 double knockouts also failed to reveal an increase in mitochondrial mutagenesis due to 8-oxo-dG damage [60]. These observations at first seem to contradict reports that overexpression of superoxide dismutase [77] or the ectopic expression of a

mitochondrially targeted catalase extends lifespan and general health in mice [78–80]. However, these studies may be telling us that mitochondrial components other than mtDNA are actually the targets of oxidative damage to the cell.

3.2. Mutational signatures of DNA damage

The discussion of mutations, SNPs or alleles in the literature often tends to name mutations relative to only one strand of the DNA, either the strand appearing in their reference sequence, or the sense strand of the gene or element under study. However, it is often not immediately possible to tell which strand the mutation arose on (C to T on the sense strand or G to A on the antisense). To not imply the causative base in the mutation it is common to refer to the basepair, limiting the mutational profile to two types of transition mutations (C:G pairs mutating to T:A, or T:A pairs to C:G), and four classes of transversion mutations (C:G to A:T, C:G to G:C, T:A to A:T and T:A to G:C).

Unfortunately, the most common mutation in mtDNA (C:G to T:A) is not diagnostic of the source of the mutation, as it may arise by various mechanisms. Hydrolytic deamination of cysteine is quite prevalent in the cell, and the resulting uracil is used as a template for an A addition on the opposing strand [81]. An identical mutational signature can also be induced by oxidative deamination of the cysteine, to create 5-hydroxyuracil, which also serves as the template of A [82]. These lesions have been demonstrated to be miscopied by pol γ, both in vitro and in vivo [83]. Additionally, oxidative deamination of guanine on the other strand to xanthine or oxanine can mispair with T when copied by other polymerases [84]. However, the same mutational profile can also occur due to replication errors. One study of pol γ errors that arose during in vitro replication showed that more than 84% of the observed mutations were (C:G to T:A) transitions despite G and C only occupying 28% of the sites in the assayed region [83]. Unfortunately these numbers cannot be seen as the definitive in vitro mutation profile, as the experimental design was such that only high-frequency mutations had their identity determined by sequencing [83].

The signature of oxidative damage is therefore sought by the effect of the rate of transversions; particularly the rate of G:C to T:A transversions via an 8-oxo-dG lesion. These lesions quite readily occur in DNA, and can even occur during DNA exposure to phenol, such as during DNA extraction [85]. Due to the prevalence of this lesion, and the unusual transversion bias that it can leave, the G:C to T:A is the signature of oxidative damage most sought during mutational studies.

3.3. Replication errors

The mutagenic capacity of DNA replication is often overlooked when discussing mtDNA mutations. The small, multi-copy nature of the mtDNA means that each base is replicated more often than its nuclear counterparts during the life of the organism, increasing the likelihood that a given site will eventually be mis-copied. After starting life as an oocyte containing only ~2×10^5 mtDNAs (~3×10^9 bp), an adult human is estimated to contain about 4×10^{13} nucleated cells [86]. With about 10^4 mtDNAs per cell, this represents ~7×10^{20} bp of human mtDNA in the body, providing ample opportunity for replication errors during development.

Fortunately, in vitro assays of the error rate of pol γA revealed a mutation rate of 5.6×10^{-7} mutations/bp/doubling [83], showing that the enzyme is actually quite accurate compared to other DNA polymerases. For comparison, *Pfu* on mitochondrial template in a similar assay gave an error rate of 6.5×10^{-7} mutations/bp/doubling [87]. Yet even with this impressively accurate replication system, a substantial number of mutations are expected, and once an error is introduced, the relaxed replication system of mtDNA can spread that mutation, even in terminally differentiated cells [88].

3.4. The extent of human heteroplasmy

Recently, next-generation sequencing has been applied to study somatic mtDNA mutations in humans [89–94]. As well, deletion mutations, where a circular mtDNA molecule lacking large stretches of sequence is maintained within the mtDNA pool, have been identified by next-generation sequencing [93] and digital PCR-based methods [95]. As the sequencing method itself is known to be quite error-prone, detailed error-checking strategies have been develop to attempt to counteract these biases [96,97]. Additionally, nuclear inserts of mitochondrial sequence (NUMTs [98]) are especially problematic in these sorts of short-read analyses, as it is very difficult to obtain mtDNA that is completely free of small nuclear DNA fragments [99]. Various processes have been employed to check for known NUMTs [91,100], but this problem is very difficult to overcome in silico due to the polymorphic state of NUMTS in humans [101].

Analysis of the mutational bias appears to further erode the possibility that oxidative damage is the major contributor to mtDNA mutations. An ultrasensitive next-generation sequencing method that essentially generates consensus sequences by tracking the multiple reads of the same DNA fragments has been developed [102] and applied to the study of mtDNA mutagenesis during ageing [89]. Samples obtained from very young brain autopsies revealed mutation loads at ~4×10^{-6} mutations/bp, but increase to ~2×10^{-5} mutations/bp from individuals >75 years of age. The mutation pattern revealed a strong bias towards the two forms of transition mutations (C:G → T:A and T:A → C:G). T:A–C:G mutations are not a predicted damage induced mutation, and there was no evidence of significant G:C → T:A transversion mutations predicted by 8-oxo-dG damage, implying replication transition errors as the source of the mutations. Additionally, the mutational bias did not shift towards damage signatures such as G:C → T:A transversions with age, implying that acquired oxidative-damage induced mutations were not accumulating over the lifetime.

An unusual strand bias in vertebrate mitochondrial DNA has long been noted. Mammalian mtDNAs have long been known to have a misbalance of nucleotides on the two strand of the mtDNA, leading to their property being separable into a heavy and a light strand on alkaline cesium chloride gradients [103]. The reference strand for the human mitochondrial DNA (the light strand) encodes ~31% C, but only ~13% G. Recent analysis of the somatic mutational spectrum in human brains revealed a mutational bias that would explain such a pattern. Throughout the coding regions, the mutational bias obtained from young human brain samples revealed that C:G to T:A mutations were more likely to occur if the light strand encoded a G at the mutated position [89]. In the samples from aged individuals, this pattern became even more prevalent, and was accompanied by a bias of T:A to C:G mutations that were more likely if the T was found on the light strand, mirroring the preference of the light strand for A (~31%) over T (~25%). However, this strand asymmetry bias was absent in the mtDNA's control region. This pattern was also reported in a subsequent next-generation sequencing study, also in the human brain [93], and had previously been shown on a small-fragment analysis of a human lung epithelium mutations. In this study, 5 of the 7 G sites underwent this transition mutation but none of the 13 C sites were mutated, and light-strand T bases was mutated 3 times more than light-strands A bases [83]. At present, the cause of this mutational bias is speculative, but the similarity to the bias observed over evolutionary timescales has been noted [89]. This may link to process to the mechanism of mtDNA replication, as inverted control region and replication elements within some fish lead to a reversal of the strand bias [104,105].

It appears that this strand-specific mutational bias against the minority nucleotides within the mtDNA strand has important functional consequences. The subsequent study of human brain noted that this strand bias produced mutations that were predicted to have a higher probability of being detrimental to the encoded protein [93]. Such a pattern makes sense, as bases conserved in the face of a strong mutational

pressure are likely to be the bases preserved by purifying selection on the resulting gene product. Additionally, these mutations will also be more likely to reoccur within an individual, and in different individuals, providing a recurring set of mutations that are likely to cause harm, if they can clonally expand to sufficient levels within the cell.

3.5. From mutation to malfunction

These new results show a surprising level of mtDNA heteroplasmy in humans, with some authors conceding that mtDNA heteroplasmy is universal in us [92]. While many of these mutations are probably harmless, some would have the potential lead to functionally deficient mitochondria, if the mutation can become the dominant allele within that cell. The pathogenic nature of mtDNA mutations in the human colon have long been analyzed and studied, where the biology of the colonic crypts allows for the clonal expansion and preservation of cells revealing mitochondrial deficiency [106,107]. These studies clearly demonstrate that mutations leading to deleterious consequence are present within us, and with sufficient time and misfortunate amplification of the deleterious alleles by the relaxed replication system, a deleterious allele can come to dominate in the cell and lead to mitochondrial dysfunction in vital organ systems.

An early modelling study clearly showed that the mutations would have to have developed early in life to have the time to reach dominating levels within the cell [108]. In agreement with this prediction, recent work in the human colonic crypts that reveal an increase in the number of dysfunctional cell clusters with age, but in the absence of an increase in the total mutational burden in the tissue. This argues that the proliferation of pre-existing mutations from early in life, not the introduction of new mutations, leads to the observed mitochondrial dysfunction [109]. One recent study with deletion mutations of mtDNA also found that the diversity of these molecules did not appear to change in samples from people of different ages [95], implying that these deletions are also generated predominantly in early life and require time to expand to become prevalent in the tissues. However, this interpretation of the deletion data has met with some controversy [110,111].

Rodents have long been the mammalian laboratory models of choice, so it was natural that ageing research would also utilize mice. The ageing mouse model was supported by a very early observation of the increase in deletion and insertion mutations in mice over their lifespan [112], similar to observations in humans. Unsurprisingly, there is not perfect concordance between natural ageing in mice and humans. Humans, with ~40× longer lifespan, will allow more time for clonal expansion and the resulting respiratory chain dysfunction. Modelling has shown that this may limit the utility of some short-lived species in their use to study the clonal expansion cycle of mtDNA mutation, as there is insufficient time for a given spontaneous mutation to reach high relative levels and lead to mitochondrial dysfunction [113]. These models are supported by recent work showing much less clonal expansion in the colonic crypts of aged mice in comparison to aged humans [114]. However, using mice with an approximately 10× increased rate of mtDNA mutation [115] appeared to speed up the process, leading to a better approximation of the cellular levels of mitochondrial dysfunction observed in the colonic crypts of humans [116]. These observations suggest that clonal expansion of the deleterious mutation to levels that can compromise mitochondrial function is critical in this process. Mutations arising late in life may simply have insufficient time to reach to the levels necessary to compromise the cell. Conversely, the earlier in life a mutation occurs, the more chance it has to clonally expand and contribute to cellular pathology.

3.6. Inherited mtDNA mutations

We typically assume that the somatic mtDNA mutations are specific to that individual, but the recent data analysis has clearly shown that mtDNA mutations can be transmitted between generations [90,109]. Indeed, most cases of mtDNA mutation related mitochondrial disease are cases where a healthy mother with non-pathogenic levels of a mutant allele gives rise to a child that crosses that mutation's heteroplasmy threshold and leads to disease [117]. In mice with increased rates of error-prone mitochondrial replication, maternal transmission of mutated mtDNAs has been shown to lead to decreased general health and fecundity [115]. Additionally, these transmitted mutations alone can reduce the lifespan of offspring with wildtype nuclear DNA [118].

Transmitted mtDNA mutations are, however, subject to some form of a female germline filtering. Work in mice has shown that the female germline can eliminate specific mtDNA mutations, especially in protein coding genes [119,120]. Work in humans has also identified that mutations that originated in the very early embryo or were derived from the oocyte also show a trend towards decreased pathogenic potential [109]. This property of mtDNA purifying selection in the germline has also been identified in *Drosophila* [121,122], implying a broad evolutionary distribution within animals. Thus, subsets of the mutant alleles in mtDNA appear to be eliminated from the germline, which would have beneficial consequences on the integrity of the offspring. However, some deleterious alleles are able to pass by this quality control system (for example in mice, see [120,123–125]). The mitochondrial germline transmission bottleneck [126] may allow for the more rapid expansion of the allele in the soma of the developing embryo, and the mutation would thus have a head start in their progress towards mitochondrial dysfunction in the ageing process.

4. Somatic mtDNA selection and drift

4.1. Seeking somatic selection of mtDNAs

It is important to stress that in nearly all instances, the clonal expansion of mtDNA mutations is best described by a neutral drift model [108], in the absence of selection for or against somatically acquired mtDNA mutations [107]. However, exceptions have been reported. Until recently, these have been confined to mitochondrial mutation levels in the blood patients with the mitochondrial disorder MELAS [127]. In the mtDNA mutator mice, the control region of the mice was found to reveal a lowered mutation load in comparison to the other, coding regions of the genome [12,128]. A subsequent study of the light strand origin of replication in mtDNA mutator mice found a similar pattern at this second replication origin, and complimentary in vitro replication assays revealed that alterations in this sequence lead to decreased replication efficiency [129]. Thus, it appears that purifying selection against mutations that decrease the replication efficiency of the mtDNA molecule is at work within the somatic tissues.

Next-generation sequencing efforts have identified other mutations with unusual behaviours in the control region. Recurring variants in the mitochondrial control region have been identified, and some appear to preferentially clonally-expand in different tissues of individuals with age [130,131]. Particularly, the position 16093 appears to confer odd segregation patterns in individuals. Two studies found that the 16093 position was heteroplasmic in other tissues, but remained homoplasmic for C in the blood [90,132]. Two additional studies found that the allele had a tissue-specific elevation in relative levels. The 16093T allele was strongly biased in the skeletal muscle, while the 16093C allele remained dominant in the other tissues sampled from the same individual [130,131]. Further investigation of this site and any others is needed, but if such tissue-specific selection is at work, deleterious mutations that occur on the same molecule as such a mutation may be drawn to high levels via a hitch-hiking effect with these otherwise harmless alleles.

4.2. Selection in tumour mtDNA

Somatic level purifying selection of mtDNA mutations has also been reported in tumour cells. One study into colorectal cancers showed that while pre-cancerous adenomas had similar mtDNA mutation frequencies

to healthy tissue, the tumours from a given patient actually had lower levels of overall mtDNA mutations than the control tissues from the same individual [133]. Though clonal propagation of a single cell may explain a decrease in the mutations present in the sample, the fact that this trend was isolated to tumours, and not present in pre-cancerous adenomas, implied a selective loss of mutations only in the cancerous samples [133]. The largest study of cancer mtDNA mutations looked at 1645 tumour samples and identified 1907 mutations specific to the tumour cells [134] Intriguingly, the mutation bias across all of the identified mutations in all tumour types was quite similar, and reflected a transition bias that again implied replicative errors and not DNA damage as the source of the mtDNA mutations [134]. Most of these mutations appeared to be segregating under a neutral drift model, except for protein-truncating mutations. These mutations, while present at the expected frequency, appeared to be limited in the relative levels that these mutations were able to obtain. A similar bias against protein-truncating mutations was previously observed in a mouse with a frameshift mutation in the *mt-nad6* gene [120]. The study also reported no anticodon-disrupting mutations within the tRNA genes, despite the expectation of observing ~8 such mutations in a study of this size. While these results conflict with claims of positive selection in previous next-generation sequencing studies of tumour mtDNA mutations [135], this study had a much larger set of mutations to use in their statistical analysis, and they applied a more stringent and evolutionarily sound null hypotheses when testing for selection within the pool of observed mutations.

4.3. Selecting for mtDNA mutations

Curiously, one of the above-mentioned studies of normal human tissues reported an excess of tissue-specific heteroplasmic mutations within liver samples. Analysis of these mutations revealed a liver-specific excess of non-synonymous heteroplasmic changes [131]. The observation of excess non-synonymous mutations is the hallmark of positive selection — the favouring of the observed amino-acid changes or linked alleles on the same mtDNA. Such a surprising finding will need further validation in subsequent studies. Intriguingly, during a next-generation sequencing analysis of mtDNA mutator mice, a single mouse showed three heteroplasmic mutations in the liver, representing more than 60% of the obtained reads, while all other mutations in the study were observed in less than 30% of the reads [99]. The three mutations were most likely linked, and represented a synonymous mutation in *mt-nad2* (c4135T), an S–L mutation in *mt-nad6* (g14042A), and a control region mutation near the ETAS2 conserved sequence block (c15579T).

5. Summary

Our expanded understanding of mitochondrial biology has led to a reassessment of the mitochondrial genome. Far from being a genome helpless to stave of the assault of oxidative damage, the mitochondria appear to have packaged [24,25] and sequestered the mtDNA away from the sites of active radical oxygen production [29]. Research continues to increase the catalogue of repair enzymes that are targeted to the mitochondria to deal with potentially mutagenic base damage [68], and the mitochondrial DNA polymerase itself appears to be highly accurate and may be able to suppress mutations arising from oxidative damage [74,83]. The application of next-generation sequencing to the study of mtDNA mutations has revealed a mutation bias of both C:G → T:A and T:A → C:G transitions; a mutational signature more consistent with replication errors than oxidative DNA damage induced mutation [60,89,134].

The mtDNA that leads to age-related mitochondrial dysfunction appears to have arisen quite early in the life of the organism, and in the majority of cases, slowly underwent neutral drift until reaching high levels and revealing their pathogenic nature [107–109]. These mutation may have occurred after the germline purifying selection has acted, thereby avoiding a "selective sieve" that may have eliminated them [119,121,122], but then have a longer journey from the single novel mutation to the predominant allele in the cell or cells. Or the mutations were maternally transmitted, having properties that hid them from the germline selection, but gave them a substantial head start by segregating them into a larger number of developing cell lineages, and potentially boosting their relative numbers in the mitochondrial germline bottleneck.

Intriguing, but rare mutations that appear to confer a replicative advantage may also complicate and exacerbate the ageing process if they carry with them a linked, deleterious allele [130,131]. And despite the emerging fields of mitochondrial quality control systems, the mtDNA itself appears to not benefit from such systems, and the pathogenic mutations appear to, in the soma, act as though they are neutral until they cross their specific heteroplasmic threshold and lead to mitochondrial dysfunction [109,116]. The soma does appear to be able to select against mutations that hinder mtDNA replication [129], or protein-truncating frameshift mutations (at least in one mouse model [120] and in human tumours [134]). While hints of alleles that confer positive selection to somatic mtDNAs have been published [131,135], these observations are new, novel, and in need of further study and verification. While it is often difficult for us to accept that our cellular systems are so influence and hindered by stochastic errors, it would be even more frightening to consider deleterious mtDNA mutations that are at a selective advantage within our somatic cells driving us more quickly through the ageing process.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2015.06.001.

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